PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Mulligan *et al.*

Serial No.: 08/252,710 Group Art Unit: 1805

Filed: June 2, 1994 Examiner: G. Elliott

For: RETROVIRAL VECTORS USEFUL FOR GENE THERAPY Attorney Docket No.: 8141-113

DECLARATION UNDER 37 C.F.R §1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Lawrence Cohen, declare and state that:

1. I am an employed by Somatix Therapy Corporation as Vice President of Research. I have worked at Somatix Therapy Corporation since 1988. My Curriculum Vitae is presented herewith as Exhibit 1.

2. I am familiar with the Specification and Claims of the above-identified patent application (heretofore the "present application") and the Office Action mailed April 17, 1996 (Paper No. 12).

3. It is my understanding that the Examiner contends that the Claims of above-identified application are obvious over the teaching of Cone and Mulligan, 1984

(Proc. Natl. Acad. Sci. *81*:6349-6353) in combination with other references.

4. I have read and am familiar with the above-identified reference by Cone and Mulligan ("Cone").

5. It is my opinion that, given the teaching in the Cone reference, one of ordinary skill in the art as of the filing date of the present application would not have had a reasonable expectation of successfully practicing the claims of the present application. My opinion is based on the fact that subsequent studies have shown that retroviral titers of the concentration disclosed by Cone (">10^5") are insufficient to effectively transduce mammalian cells without selection. For example, to my knowledge the first published report that primary human tumor cells could be transduced without selection was made in 1993 by Jaffee *et al.*, 1993, Cancer Research *53*:2221-2226 ("Jaffee", attached as Exhibit 2). Jaffee used a novel retroviral vector that is an embodiment of the vectors described in the above-identified application. To my knowledge, retroviral transduction without selection (as reported in Figure 1 of Exhibit 2) generally requires high titer stocks of transducing virus. In particular, a <u>minimum</u> titer of approximately <u>5×10^6</u> transducing virus per ml is required. This concentration of retrovirus is at least several fold higher (if not a full order of magnitude higher) than the retroviral titers reported by Cone. Accordingly, it is

my opinion that one of ordinary skill could not have used the teaching of Cone to practice the presently claimed invention.

6. It is also my opinion that, prior to the present invention, the cell lines and vectors taught by Cone would have not provided one of ordinary skill with a general expectation that mammalian cells could be successfully transduced without the use of selectable markers. In fact, the first published disclosure of human primary tumor cells transduced without selection (i.e., Jaffee *et al.*) was made nine years after Cone was published. During those nine years, novel retroviral packaging cell lines were constructed (as described in U.S. Patent No. 5,449,614 "'614", issued September 12, 1995), and the equally novel retroviral vectors of the present application were constructed. Jaffee *et al.* obtained these pioneering results after combining novel vectors and a packaging cell line that were both developed well after Cone was published. It should also be noted that one of the incentives for producing the claimed vectors and the packaging cells described in the '614 patent was the realization that many methods of *ex vivo* or *in vivo* gene therapy would not be compatible with the prolonged periods of selective culture used in previous methods of transduction (including the methods specifically described by Cone). In many respects, the above insight played a key role in motivating the development of retroviral vectors that lack a selectable

marker (i.e., the presently claimed vectors). Cone neither taught nor suggested the above insight.

7. I hereby further declare, under penalty of perjury under the laws of the United States of America, that all statements made herein of my own knowledge are true and that all statements made upon information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 10/11/96

By: _____
Lawrence Cohen, Ph.D.

Exhibit A

Curriculum Vitae
LAWRENCE K. COHEN, Ph.D.

PERSONAL:

Address: 5670 Cabot Drive
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EDUCATION

1974 B.A., Grinnell College
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1976 M.S., University of Illinois
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1981 Ph.D., University of Illinois
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POSTDOCTORAL TRAINING:

1981-1983 **RESEARCH FELLOW IN MICROBIOLOGY AND MOLECULAR GENETICS,**
Dana Farber Cancer Institute,
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1983-1985 **RESEARCH FELLOW IN BIOLOGICAL CHEMISTRY,**
Harvard Medical School, with Bryan E. Roberts

AWARDS:

1981 American Cancer Society Fellowship

1982-1985 NIH, National Research Service Award

1985-1986 Medical Research Foundation Grant

PROFESSIONAL EXPERIENCE:

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Somatix Therapy Corporation
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1992-1993 **DIRECTOR, MOLECULAR BIOLOGY**
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1991-1992 **MANAGER, MOLECULAR BIOLOGY**
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1988-1991 **PROGRAM LEADER**
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1987-1988 **SENIOR SCIENTIST AND GROUP LEADER**
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Applied bioTechnology, Inc.
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1986-1987 **RESEARCH SCIENTIST**
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BIBLIOGRAPHY:

1. Cohen, L.K., Leuking, D.R. and S. Kaplan. Intermembrane phospholipid transfer mediated by cell-free extracts of Rhodopseudomonas sphaeroides. J. Biol. Chem. 1979; **254**:721-728.

2. Lynn, S.P., Cohen, L.K., Gardner, J.F. and S. Kaplan. Characterization of a site specific restriction endonuclease from Rhodopseudomonas sphaeroides. J. Bacteriol. 1979; **138**:505-509.

3. Lynn, S.P., Cohen, L.K., Kaplan, S. and J.F. Gardner. RsaI: a new sequence-specific endonuclease from Rhodopseudomonas sphaeroides. J. Bacteriol. 1980; **142**:380-383.

4. Cohen, L.K. and S. Kaplan. The non-detergent solubilization and isolation of intracytoplasmic membrane polypeptides from Rhodopseudomonas sphaeroides. J. Biol. Chem. 1981;**256**:5901-5908.

5. Cohen, L.K. and S. Kaplan. Characterization of the three major intracytoplasmic membrane polypeptides from Rhodopseudomonas sphaeroides. J. Biol. Chem. 1981;**256**:5909-5915.

6. Edson, C.M., Cohen, L.K., Henle, W. and J.L. Strominger. An unusually high-titer anti-Epstein Barr virus (EBV) serum and its use in the study of EBV-specific proteins synthesized *in vitro* and *in vivo*. J. Immunol. 1983;**130**:919-924.

7. Miller, J.S., Paterson, B.M., Ricciardi, R.P., Cohen, L.K. and B.E. Roberts. Methods utilizing cell-free protein-synthesizing systems for the identification of recombinant DNA molecules. Methods in Enzymol. 1983;**101**:650-674.

8. Cohen, L.K., Speck, S.H., Roberts, B.E. and J.L. Strominger. Identification and mapping of polypeptides encoded by the P3HR-1 strain of Epstein-Barr virus. Proc. Natl. Acad. Sci. USA. 1984;**81**:4183-4187.

9. Morgan, J.R., Cohen, L.K. and B.E., Roberts. Identification of the DNA sequence encoding the large subunit of the mRNA capping enzyme of vaccinia virus. J. Virol. 1984;**52**:206-214.

10. Spyropoulos, D.D., Roberts, B.E., Panicali, D.L. and L.K. Cohen. Delineation of the viral products of recombination in vaccinia virus-infected cells. J. Virol. 1988;**62**:1046-1054.

11. Spyropoulos, D.D., Stallard, G., Roberts, B.E., Panicali, and L.K. Cohen. The utilization of DNA recombination for the two step replacement of growth factor sequences in the vaccinia virus genome. J. Virol. 1991;**65**:4609-18.

12. Jenkins, S., Gritz, L., Fedor, C.H., O'Neill, E.M., Cohen, L.K., and D. L. Panicali. Formation of lentivirus particles by mammalian cells infected with recombinant fowlpox virus. AIDS Res. Hum. Retroviruses 1991;**7**:991-998.

13. Lee, M.S., Roos, J.M., McGuigan, L.C., Smith, K.A., Cormier, N., Cohen, L.K., Roberts, B.E., and L.G. Payne. Molecular attenuation of vaccini virus; mutant generation and animal characterization. J. Virol. 1992;**66**:2617-2630.

14. Smith, K.A., Stallard, V., Roos, J.M., Hart, C., Cormier, N., Cohen, L.K., Roberts, B.E. and L.G. Payne. Host range selection of vaccinia recombinants containing insertions of foreign genes into non-coding sequences. Vaccine 1993; **11**:43-53.

15. Jaffee, E.M., Dranoff, G., Cohen, L.K., Hauda, K.M., Clift, S.M., Marshall, F.F., Mulligan, R.C. and D.M. Pardoll. High efficiency gene transfer into primary human tumor explants without cell selection. Cancer Res. 1993; **53**:2221-2226.

16. Sanda, M.G., Ayyagari, S.R., Jaffee, E.M., Epstein, J.L., Clift, S., Cohen, L.K., Dranoff, G., Pardoll, D.M., Mulligan, R.C. and J.W. Simons. Demonstration of a rational strategy for human prostate cancer gene therapy. J. Urol. 1994; **151**:622-628.

17. Varavani, J.D., Belloni, B., Tarlochan, N., Smith, J. Couto, L., Rabier, M., Clift, S., Berns, A. and L.K. Cohen. Gene therapy for Hemophilia A: Production of therapeutic levels of human factor VIII *in vivo* mice. Proc. Natl. Acad. Sci. USA 1995; **92**: 1023-1027.

18. Berns, A.J., Clift, S., Cohen, L.K., Donehower, R.C., Dranoff, G., Hauda, K.M., Jaffee, E.M., Lazenby, A.J., Levitsky, H.I., Marshall. FF. Phase I study of non-replicating autologous tumor cell injections using cells prepared with or without GM-CSF gene transduction in patients with metastatic renal cell carcinoma. Human Gene Therapy 1995; **6**:347-368.

PATENTS

L.K. Cohen and D.L. Panicalli. (1992) Recombinant fowlpox virus and recombination vector. U.S. Patent #5,093,258.

L.K. Cohen, B. Guild, L.F. Rayfield, P. Robbins and R.C. Mulligan. Retroviral vectors useful for gene therapy. U.S. Patent Application #07-786015, Filed 10/31/91

Exhibit 2

High Efficiency Gene Transfer into Primary Human Tumor Explants without Cell Selection[1]

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Abstract

Preclinical studies with murine tumor models have demonstrated that autologous tumor cell vaccines engineered to secrete certain cytokines in a paracrine fashion elicit systemic immune responses capable of eliminating small amounts of established tumor. These results have engendered much interest in developing this strategy for gene therapy of human cancer. The major limitation to creating genetically modified autologous human tumor vaccines is efficient gene transfer into primary tumor explants, since the majority of human tumors fail to proliferate in long-term culture. Using the retroviral vector MFG in conjunction with short-term culture techniques, we have achieved, in the absence of selection, a mean transduction efficiency of 60% in primary renal, ovarian, and pancreatic tumor explants, and we have developed an autologous granulocyte-macrophage colony-stimulating factor secreting tumor vaccine for clinical trials.

Introduction

Recently, a new tumor vaccine approach using genetically altered autologous tumor cells to secrete local concentrations of cytokines has been developed in murine models (1–9). In some of these models, lymphokine gene transduced tumor cells have been shown to generate a local, tumor-specific immune response when administered as a s.c. vaccination (1–3, 5–9). In addition to rejecting the genetically modified tumor cells, vaccinated animals may develop a T-cell-dependent systemic immunity which in some cases can cure micrometastases established prior to treatment with the genetically altered tumor cells (1–3, 7, 8). Recently, a direct comparison of multiple cytokine genes transferred into a poorly immunological murine melanoma model identified GM-CSF[3] as the most potent in generating a protective response (7). Two critical features of this approach include: (*a*) the ability to generate a T-cell dependent tumor-specific systemic immunity; and (*b*) the production of lymphokine only at the tumor site, thereby producing a strong antitumor immune response without systemic toxicity.

The extension of this strategy to human cancer therapy will require two technical advances. First, the gene transfer systems used must be able to routinely introduce lymphokine genes into human tumors efficiently and must be able to produce consistent levels of gene expression. Other investigators report an efficiency of transduction of no better than 1 in 100 tumor cells so that the vector carrying the cytokine gene must also transfer a selection marker.[4] Second, the tumor cells that are transduced must be from primary human tumor cultures established at the time of surgery. All previous reports of gene transfer into human tumors have used vectors containing selectable markers and stable long-term cell lines rather than primary tumor cell cultures. Because stable long-term cultures cannot be established for the vast majority of human tumor explants (melanoma being the exception), these gene transfer approaches will fail to generate sufficient numbers of genetically modified cells (10, 11).[4] Even for the rare circumstances in which long-term cell lines can be established, transduction of cell lines and posttransduction selection might result in selective loss of expression of critical tumor-specific antigens expressed by the parent tumor *in vivo*. Boon *et al.* (12, 13) have provided evidence to support this contention by showing that it is possible to isolate several tumor-specific T-cell clones from a patient with malignant melanoma. Evaluation of these T-cell clones for lysis of melanoma tumor cell clones obtained from the same patient revealed three melanoma-specific antigens. The first antigen was present on all melanoma clones tested, the second antigen was lost during long-term culture, and the third antigen was expressed on a minority of tumor clones (12, 13).

In this article we report the use of a retroviral vector system to achieve high efficiency transduction of primary human tumor explants without requiring long-term culture or selection. These results provide the basis for the routine production of genetically modified autologous tumor vaccines.

Materials and Methods

Patients. All surgical specimens were obtained from patients with a histological diagnosis of either renal cell carcinoma; ovarian carcinoma; adenocarcinoma of the lung, colon, and pancreas; squamous cell carcinoma of the hypo pharynx; or carcinoma of the breast. All of the tumors were primary resections except for the five ovarian tumors which were obtained from ascites and the two breast carcinomas which were obtained from pleural fluid. Informed consent to use these surgical specimens was obtained from all patients prior to the surgical procedure.

Dissociation of Primary Human Tumor Explants. All tumors were transported from the operating room on ice and were mechanically dissociated into 1–5-mm fragments within 1 h. These tumor fragments were then enzymatically digested, initially by exposure to collagenase (GIBCO; 1 mg/ml; 173 units/mg), for 20–30 min in a vigorously shaking 37°C incubator. Single cells in the supernatant were removed. The remaining pellet was exposed to two more cycles of enzymatic digestion with collagenase followed by trypsin-EDTA (GIBCO; 0.25% trypsin, 1 mM EDTA) and DNase I (776785; 0.1 mg/ml; Boehringer Mannheim Biochemical) until all of the fragments were fully digested. This process yields approximately 5×10^6 viable malignant cells

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[1] This work was supported in part by NIH Training Grant C809071, and Core Grant C806973 and by the Somatix Therapy Corporation. D. M. P. is a recipient of the Cancer Research Institute-Benjamin Jacobson Family Investigator Award and the RJR Nabisco Research Scholars Award.

[2] To whom requests for reprints should be addressed, at Johns Hopkins Oncology Center, Johns Hopkins School of Medicine, 720 Rutland Avenue, Ross Building, Room 364, Baltimore, MD 21205.

[3] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

[4] S. A. Rosenberg, W. F. Anderson, M. R. Blaese, S. E. Ettinghausen, P. Hwu, S. I. Karp, A. Kasid, J. J. Mule, D. R. Parkinson, J. C. Salo, D. J. Schwartzentruber, S. L. Topalian, J. S. Weber, J. R. Yanelli, J. C. Yang, and W. M. Linehan. Immunization of cancer patients using autologous cancer cells modified by insertion of the gene for interleukin-2, Recombinant DNA Advisory Committee Protocol, approved 3/1/92.

from a 1.0-g tumor mass. Cells from all digested fractions were pooled and incubated in selected growth medium. Cells were passaged when each flask reached 80–100% confluence.

***In Vitro* Growth of Primary Human Tumor Explants.** Conditions necessary for the short-term growth of primary human tumor cultures were evaluated in the following way. Freshly digested tumor cells were plated in duplicate at 1×10^5 cells/75 cm^2 flask. Each growth condition was evaluated both separately and in combination with other growth supplements (Tables 1 and 2). Different media including RPMI, Dulbecco's modified Eagle's medium, Ham's and Aim-V preparations, and lots of FBS were the initial components of growth medium screened (Tables 1 and 2). Following identification of the optimal medium and serum, additional additives were systematically evaluated (Table 2). Each supplement was evaluated for at least 2 *in vitro* passages/patient tumor and for enhancing the growth of at least 2 different patients' tumors of before routinely including it as a supplement for tumor growth. When tumor cells in each flask reached 100% confluence, they were trypsinized and counted before being replated.

Transduction of Primary Human Tumor Cultures. Transduction is performed with the MFG retroviral vector system. The structure of MFG has recently been described (7, 17). Briefly, in this vector, Moloney murine leukemia virus long terminal repeat sequences are used to generate both a full length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA (analogous to the Moloney murine leukemia virus *env* mRNA) which is responsible for the expression of inserted sequences. The vector retains both sequences in the viral gag region shown to improve the encapsidation of viral RNA (16) and the normal 5′ and 3′ splice sites necessary for the generation of the *env* mRNA. Protein coding sequences are inserted between the *Nco*I and *Bam*HI sites in such a way that the initiation codon of the inserted sequences is placed precisely at the position of the viral *env* initiation codon, and minimal 3′ nontranslated sequences are included in the insert. No selectable marker exists in the vector. Complementary DNA sequences encoding the cytokine were inserted into MFG and the resulting vector constructs were introduced into CRIP cells as previously described (17) in order to generate recombinant virus with amphotropic host range.

The retroviral producer cell line, CRIP, is grown in culture to confluency in Dulbecco's modified Eagle's medium + 10% calf serum. Two days prior to transduction, the cells are trypsinized and replated at a density of 2×10^6 cells/100-mm culture dish. One day prior to transduction, 10 cc of fresh medium are added to the cells. On the day of transduction, a 24-h supernatant is collected and filtered through an 0.45-μm filter to remove contaminating retroviral producer cells. Human tumor explants growing in culture at a density of 5×10^5 cells/75 cm^2 culture dish are incubated with 10 ml of retroviral supernatant containing 70 μg/ml of DEAE-dextran (Sigma, St. Louis, MO; D-9885) at 37°C. (Of note, it is important to perform a dose response curve to compare new preparations of DEAE-dextran prior to use because we have found that the optimal concentration of DEAE-dextran needed for successful transduction can vary between preparations.) A 4-h incubation period is optimal for efficient gene transfer. Following transduction, the retroviral supernatant is removed and the cells are grown in culture for an additional 3 days to allow for integration and expression of the transferred DNA.

Assay for *LacZ* Expression. To determine the rate of transduction of primary human tumor explants using the MFG retroviral vector system, we used a vector containing the *LacZ* marker gene. This gene encodes for expression of *Escherichia coli* β-galactosidase and is used because it is easy to assay for its expression using cytochemistry methods (18).

Three days after infection with the retroviral vector, adherent tumor cells are trypsinized and washed with PBS (pH 7.3). The tumor cells are then fixed with gluteraldehyde (final concentration of 0.5% gluteraldehyde in PBS) for 5 min on ice. The cells are subsequently washed once with PBS and resuspended in a substrate solution containing the substrate bluogal at a final concentration of 300 μg/ml, 0.2% of a 1 M solution of $MgCl_2$, 0.16% potassium ferricyanide, and 0.2% potassium ferrocyanide in PBS (pH 7.3). The tumor cells are incubated overnight at 37°C without CO_2. Following incubation, the tumor cells are counted on a hemocytometer. The transduction efficiency is defined as the percentage of positively stained cells. At least 200 cells are counted/specimen. Cells are determined to be positive for the transferred gene if they have a cytoplasm that appears to be uniformly blue.

Assay for Human GM-CSF Production. The TF-1 cell line, described by Kitamura *et al.* (19), is a human line isolated from a patient with erythroleukemia and is used to evaluate GM-CSF production by transduced human tumor cultures. Growth of these cells is dependent on the addition of GM-CSF to their media. These cells are passaged 3 times/week in RPMI 1640 supplemented with 10% FBS, 1 mM L-glutamine, 50 μg/ml penicillin and streptomycin, and 5 ng/ml of recombinant human GM-CSF. A [^{3}H)]hymidine incorporation proliferation assay using these cells is performed by first washing these cells 3 times to remove GM-CSF. The experimental supernatants obtained by growing 1×10^5 transduced human tumor cells for 24 h in 5 cc of tumor growth medium are collected and filtered through an 0.45-μm filter to remove contaminating cells and plated (100 μl/well) in 96-well flat plates at 1:3 dilutions. 1×10^4 TF-1 cells (100 μl/well) are added to each well in growth medium without GM-CSF and are incubated × 24 h at 37°C.

[^{3}H]thymidine (1 μCi) is added to each well and the cells are incubated for an additional 18–24 h. At the end of the incubation period the cells in each well are harvested and counted in a beta counter.

Results

Establishing Primary Human Tumor Cultures. Our method of digesting freshly excised tumor specimens routinely yields 5×10^6 viable tumor cells/g of excised tumor. It should be pointed out that

Table 1 *Basal media and FBS screened for primary human tumor growth support in vitro*

At least 2 histologically similar tumor cell types were grown in each of the basal media listed above and studied for enhanced *in vitro* proliferation. Varying amounts of FBS were also evaluated. Growth rate was initially evaluated by daily observation and scoring of duplicate flasks.[a]

Medium	Renal cell carcinoma	Ovarian carcinoma	Pancreatic and colonic carcinoma	Breast carcinoma	Adeno lung and SCCA[b, c]
Base medium					
RPMI 1640	++[c]	++	++	–	++
Ham's F10	++	++	NT	–	NT
Modified Eagle's	–	–	NT	NT	NT
Aim-V	–	–	NT	–	NT
DMEM[d] Ham's F12	NT	NT	NT	++	NT
Defined FBS (%)					
0	–	–	–	–	NT
5	–	–	–	+++	NT
10	–	–	–	++	NT
15	++	++	++	++	NT
20	++++	++++	++++	NC	++++
25	++	++	NT	NT	NT

[a] Cultures were scored using the following scoring system: NC, no change in growth rate; –, decreased growth; ++, 2-fold increase in growth; +++, 3-fold increase in growth; ++++, 4-fold increase in growth; NT = not tested (a 2-fold increase in growth = 2 [times] the number of cells obtained from the control flask during a 96-h incubation period). When the cells in each flask reached 100% confluence (total cell number = approximately 2×10^6/flask), they were trypsinized, counted, and replated for further growth evaluation. The number of passages reached/given time period was also recorded. For some tumors the doubling time was estimated using a [^{3}H]thymidine uptake proliferation assay.

[b] Adenocarcinoma (Adeno) of the lung and squamous cell carcinoma (SCCA) of the tonsil.

[c] Only one tumor of each tested.

[d] DMEM, Dulbecco's modified Eagle's medium.

Table [redacted] *Growth supplements screened for primary human tumor growth s[redacted] in vitro*

At least 2 histologically similar tumor cell types were studied for enhanced *in vitro* proliferation using each of the growth supplements listed above. All supplements were added to the basal medium and FBS selected for optimal tumor growth (Table 1). Growth rates of duplicate flasks of tumor cells were evaluated daily using the scoring system described in the methods section and in Table 1. When the cells in each flask reached 100% confluence, they were trypsinized, counted, and replated for further growth evaluation. The number of passages reached/given time period was also recorded (data not shown). For some tumors the doubling time was estimated using a [^{3}H]thymidine uptake proliferation assay.

Growth supplement	Renal cell carcinoma	Ovarian carcinoma	Pancreatic and colonic carcinoma	Breast carcinoma	Adeno lung and SCCA[b,c]
Recombinant GF[c]					
Human insulin	NC	++++	++++	++	++
Epidermal GF	++	++	NC	++	++++
Fibroblast GF	NC	NC	NT	NT	NT
Insulin-like GF-1	NC	++	NC	NC	NC
Insulin-like GF-2	NC	NC	NC	NC	NC
IL-6	NC	NC	NT	NT	NT
IL-3	NC	NC	NT	NT	NT
Keratinocyte GF	++	++	NT	NT	NT
Insulin-like GF 1 + 2	++	++	++	NT	NT
Keratinocyte + Epidermal GF	++	+++	NT	NT	NT
Other supplements					
Hepatocyte GF	+++	NC	++	NT	NT
BPE	++	NC	++	++	NT
Hydrocortisone	++	NC	++	++	NT
Ascorbic acid	NT	NT	–	++	NT
Keratinocyte + Hepatocyte GF	++	NC	++	NT	NT
Matrigel	NC	NC	NT	NT	NT
Selenium + Transferrin	NC	+++	+++	+++	+++
Triiodothyronine	NT	NT	–	++++	NT
Glucagon					
Estradiol					
Ethanolamine					
Phosphoethanolamine (14)					
TPB (15)	++++	NC	NT	NT	NT

[a] Adenocarcinoma (Adeno) of the lung and squamous cell carcinoma (SCCA) of the tonsil.
[b] Only one tumor of each tested.
[c] GF, growth factors; BPE, bovine pituitary extract; TPB, tryptose phosphate broth.

these results take into account our recent data which suggest that mechanical dissociation into 5-mm tumor fragments prior to digestion is superior to enzymatically digesting smaller tumor fragments, especially when only collagenase is used for the initial enzymatic digestion.[5] In fact, when tumor cells that are mechanically dissociated are grown separately from collagenase-digested tumor cells but in the same growth medium, the initial growth rate of the mechanically dissociated tumor cell population is much slower, resulting in roughly one-half the number of expanded cells during the first two *in vitro* passages (data not shown).

We have evaluated 24 renal cell carcinomas, 26 ovarian carcinomas, 8 colon carcinomas, 5 pancreatic carcinomas, 3 breast carcinomas, an adenocarcinoma of the lung, and a squamous cell carcinoma of the tonsil for short-term *in vitro* growth. Growth conditions were studied using the procedure described in "Materials and Methods." Initially, the optimal base medium and FBS were determined for each histological tumor type (Table 1). Lots of characterized and defined FBS were screened. Once an adequate lot of serum was identified, the percentage of FBS was evaluated. A list of the base media and percentage of defined fetal bovine sera is shown in Table 1. Additional supplements were subsequently evaluated. A list of these growth supplements can be found in Table 2. Optimal short-term growth of fresh human tumor explants is dependent on several conditions. Common to all histological tumor types with the exception of breast carcinoma is the percentage of high grade FBS used in the buffered medium. All of the other tumors grown in our laboratory to date grow well in 20% characterized or defined FBS. Breast carcinomas require 5% FBS or less. Greater than 5% FBS can result in overgrowth of fibroblasts (14). Ovarian, colon, and pancreatic tumor explants also require the addition of human insulin (0.2 units/ml). The addition of transferrin and selenium will often enhance the growth of ovarian and breast carcinomas. Renal cell carcinoma explants require the addition of tryptose-phosphate broth (10%; Difco; 0060–01–6) and occasionally, bovine pituitary extract (Sigma; P1167). Colon tumor explants grow well in a medium that is also supplemented with bovine pituitary extract. With these defined conditions an expansion of the tumor cell population of 10-fold or greater is routinely obtained during a period of 2–4 weeks (Table 3).

In addition, an attempt was made to identify characteristics of the initial tumor specimen that were associated with enhanced or inhibited *in vitro* growth. In particular, histological diagnosis, degree of malignant cell differentiation, and degree of necrosis were compared with the last *in vitro* passage achieved by the tumor. Interestingly, only the degree of necrosis adversely affected the success of short-term *in vitro* growth. In contrast, all malignant histologies could be grown equally well provided that the conditions for each histology were optimized. To illustrate this point, the results for 26 nephrectomy specimens evaluated for *in vitro* expansion are shown in Table 3.

Transduction of Human Tumor Explants. We have identified three conditions that are critical for high efficiency gene transfer to primary cultures of human tumor cells. First, successful tranduction requires a vector system that can transduce cells efficiently, resulting in consistent levels of gene expression. Although the quality of retroviral supernatants can vary, this problem is easily controlled by tittering of the retroviral supernatants using easily transducible cell lines prior to use in gene transfer to the fresh human tumor explants. Second, efficient retroviral gene transfer and expression depends on the percentage of tumor cells within the tumor population that are actively proliferating at the time of gene transfer. In general, transduction efficiency correlates with the percentage of tumor cells undergoing cell cycling since integration of the retroviral vector into the host genome is required for expression of the transferred gene. Third,

[5] A. Burns, L. Cohen, R. C. Donehower, G. Dranoff, K. M. Hauda, E. M. Jaffee, A. J. Lazenby, H. I. Levitsky, F. F. Marshall, R. C. Mulligan, W. G. Nelson, A. H. Owens, D. M. Pardoll, G. Parry, A. H. Partin, S. Piantadosi, J. W. Simons, and J. R. Zabora. Phase I study of non-replicating autologous tumor cell injections using cells prepared with or without GM-CSF gene transduction in patients with metastatic renal cell carcinoma. Recombinant DNA Advisory Committee Protocol, approved 3/1/93.

Table 3 *Results of in vitro expansion of 26 fresh human renal cell explants after nephrectomy*

All tumors were obtained at the time of surgical excision; mechanically dissociated; and enzymatically digested into a single cell suspension. Cells were grown *in vitro* using the growth conditions described in "Results." The initial cell number was usually 1×10^7 cells obtained from a 2-g tumor mass. Tumor cells were passaged every 4–5 days by trypsinizing the cells off of the culture flask and splitting them 1:3. A passage (P) was defined as the point at which the tumor cells reached 100% confluence. The *n*-fold increase represents the estimated increase in tumor cells if the total number of cells obtained at the end of each passage were continued in culture until the last passage obtained (3-fold increase = 3 100% confluent tissue culture flasks obtained as the result of splitting a single flask 1:3). The number of patient specimens (N) with a particular histological subtype that reached each *in vitro* passage is also recorded in parentheses.

Last passage	*n*-fold increase	Histological subtype (N)
P2	9	Oncocytoma (1) Clear cell, grade III (1)[a]
P3	27	Oncocytoma (2) Papillary, grade II (1)[b]
P4	81	Granular, papillary (3) Granular (1) Grade I, clear cell (1) Grade II, clear cell (1) Grade III, clear cell (1)
P5	243	Sarcomatoid (1) Grade II, clear cell (1) Grade III, multicystic (1) Papillary, grade II (1)
P6	729	Grade II, clear cell (2)
P7	2187	Grade II, clear cell (3) Papillary, clear cell (1) Sarcomatoid (1)
No growth	0	Benign histologies (2) Oncocytoma (1)

[a] Small tumor, too few cells obtained.
[b] Severe necrosis.

transduction efficiency can be enhanced by the addition of polymers to the retroviral supernatant just prior to exposure of the target cells to the retroviral vector. Enhanced gene transfer is thought to occur via a charge-mediated mechanism that affects virus binding to or penetration of the target cell. The polycations protamine, polybrene, and DEAE-dextran are routinely used for this purpose (20).[4] However, protamine, which has been commonly used for previous gene therapy trials, gives low efficiencies of gene transfer to human lymphocytes and tumor cell lines, and therefore requires the cotransfer of a selectable marker.[4] We therefore performed an extensive comparison of polybrene and DEAE-dextran, using a wide range of concentrations and exposure times of these agents to the target cell. Although both agents increased the transduction efficiency of human tumor cells in primary culture, DEAE-dextran resulted in less cell toxicity than polybrene at equivalent enhancement doses. Thus, we find that DEAE-dextran is a potent enhancer of gene transfer to primary human tumor cultures. It also results in less target cell toxicity.

An ovarian tumor cell line derived from a patient with malignant ascites was initially used to evaluate optimal transduction conditions. These conditions are described in "Materials and Methods."

These conditions were then used to evaluate 8 renal cell carcinomas, 5 ovarian carcinomas, 5 colorectal carcinomas, 2 pancreatic carcinomas, 2 breast tumors, 1 squamous cell carcinoma of the tonsil, and 1 adenocarcinoma of the lung for transduction efficiency using the MFG vector carrying the *E. coli LacZ* gene (Fig. 1*A*). Using the conditions described above, we were able to show expression of the *LacZ* marker gene in at least 20% of each tumor population, with a mean of 70% for renal cell carcinomas, 65% for ovarian carcinomas, and 43% for colon carcinomas. In 16 of 21 tumors, we were able to achieve at least a 40% transduction efficiency. We have also attempted to transduce several other histological human tumors, including a squamous cell carcinoma arising from a tonsil (40% transduction), an adenocarcinoma of the lung (82% transduction), and 2 breast carcinomas (mean transduction efficiency of 28%).

Recent studies evaluating the antitumor immune response generated by a variety of single lymphokines using the B16 melanoma murine model revealed that the cytokine GM-CSF can generate an enhanced antitumor immune response that is much greater than the response generated by any other cytokine tested (7). *In vivo* depletion studies revealed that this response is dependent on both CD4+ and CD8+ T-cells. Additional experiments indicated that maximal systemic immunity was achieved when the average level of GM-CSF production by the vaccine cells was equal to or greater than 36 ng/10^6 cells/24 h.[5] These preclinical studies provide the immunological data needed to begin to apply this approach to the treatment of cancer in patients. As a prelude to initiating clinical vaccine trials, we used the same MFG retroviral vector to transfer the human GM-CSF gene to 3 renal cell carcinomas, 2 colon carcinomas, and 3 pancreatic carcinomas (Fig. 1*B*). In 6 of the 8 tumors, GM-CSF production was at least 50 ng/10^6 cells/24 h. It was even possible to improve production of GM-CSF by the less efficiently transduced tumors, 1 to more than 50 ng/10^6 cells/24 h, after a second retroviral transduction was performed. Southern blot analysis of 5 of the renal cell carcinoma cultures genetically altered to secrete GM-CSF revealed a range of integrated vector copy numbers between 0.5 and 2 copies/cell. This correlated with a range of GM-CSF secretion between 26 and 74 ng/10^6 cells/24 h (Table 4). In addition, the transduced cells can freeze and thaw easily, with minimal loss of the number of viable, lymphokine-producing cells (Fig. 1*B*). This confirms that the MFG vector system has the ability to very efficiently transfer human cytokine genes to fresh human tumor explants.

Discussion

Preclinical murine studies have shown that tumor cells, genetically altered to secrete lymphokines, will increase the immunogenicity of a tumor when given s.c. in the form of a vaccine. The gene transfer of GM-CSF, in particular, stands out as the cytokine that generates the greatest antitumor immune response in murine models (7). Furthermore, the local secretion of GM-CSF at the site of the tumor leads to the production of both helper and cytolytic cells that can circulate and eradicate existing tumor at distant sites. In addition, our previous studies have also shown that tumor cells genetically altered to secrete local concentrations of GM-CSF will cure mice of micrometastatic melanoma. These studies therefore provide the theoretical basis for using this approach to treat human cancers.

We now report that it is technically possible to produce a genetically-altered autologous human tumor vaccine for patient trials. In addition, we provide evidence for successful gene transfer to short-term, primary tumor cultures, which is a critical advantage over previous reports of genetically altered long-term human tumor cell lines. Since the goal of a genetically altered tumor vaccine is to activate the immune system of a patient to recognize and eradicate existing tumor at other sites, therapeutic efficacy will depend on reinjecting a population of vaccine cells that represent the antigenic diversity of the parent population. There is now evidence that suggests that long-term *in vitro* culture of human tumor cell lines results in the loss of expression of relevant tumor antigens. For example, Boon *et al.* (12, 13) found that the immunodominant T-cell recognized antigen in a human melanoma was spontaneously lost upon long-term culture and subcloning. To our knowledge, this is the first report of high efficiency gene transfer to primary human tumor cultures without requiring simultaneous transfer of a selection marker for *in vitro* selection of the transduced cells.

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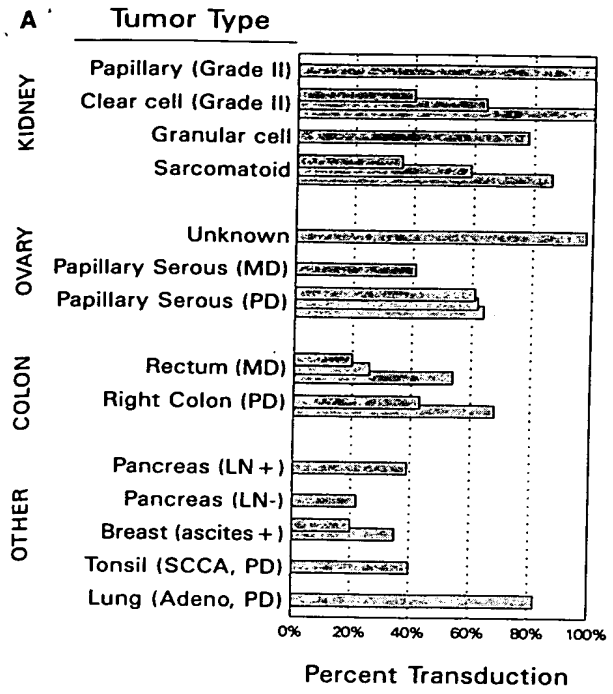

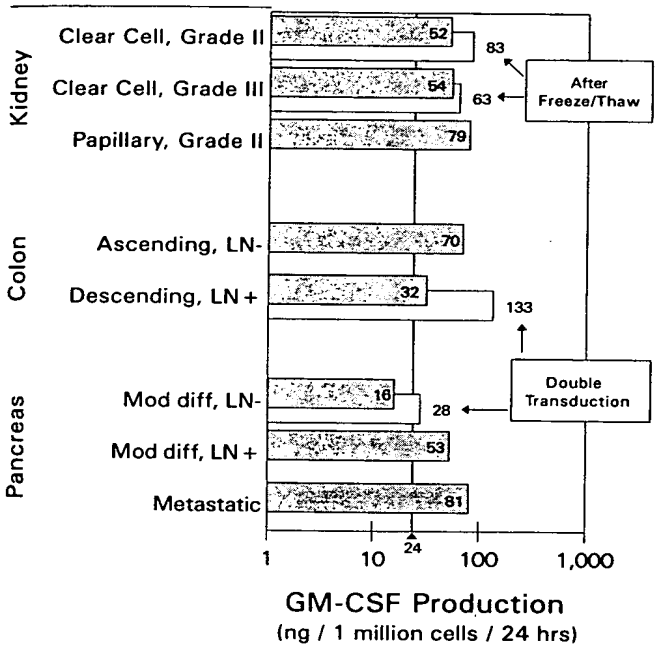

Fig. 1. *A*, transduction efficiency of primary human tumor cell populations. All tumor cells were transduced with the MFG retroviral vector carrying the *E. coli LacZ* marker gene. β-galactosidase activity was assayed using the substrate staining procedure described in "Materials and Methods." Percentage transduction was calculated as the total number of positively stained cells/200 cells stained. *MD*, moderately differentiated; *PD*, poorly differentiated; *LN*, lymph node; *SCCA*, squamous cell carcinoma; *Adeno*, adenocarcinoma. *B*, GM-CSF production by transduced renal cell, colonic, and pancreatic tumor cells. All tumor cells were transduced with the MFG retroviral vector carrying the human GM-CSF gene. GM-CSF production by 1 million transduced tumor cells over a 24-h period at least 3 days following transduction was determined using the bioassay described in "Materials and Methods." Two transduced renal tumor cell cultures were evaluated for GM-CSF production before and after freezing and thawing the tumor cells. One pancreatic and two colon tumor cultures were evaluated for GM-CSF production after two consecutive GM-CSF gene transfers. The second GM-CSF gene transfer was performed 72 h following the first gene transfer. Of note, GM-CSF production by primary untransduced tumor cultures at the corresponding *in vitro* passage was also determined. GM-CSF production was detected by 2 renal cell carcinomas (12 *versus* 83 and 3.2 *versus* 78.6 ng/10^6 cells/24 h, untransduced *versus* transduced, respectively), and 1 colonic carcinoma (20 *versus* 70 ng/10^6 cells/24 h, untransduced *versus* transduced, respectively). *LN*, lymph node. *N*-(2,3-Dioleoyloxy)propyl)-*N,N,N*-trimethyl-ammoniummethylsulfate, a cationic lipid often used for transfection of DNA into mammalian cells (available from Boehringer Mannheim), was used instead of DEAE-dextran to enhance retroviral infection. It was found to be as effective but slightly less toxic to the tumor cells when compared with DEAE-dextran, enabling a second transduction to be performed on the same cells. Although the second transduction resulted in improved GM-CSF production, its cost may prohibit its use in clinical trials.

Table 4 *Results of the comparison of GM-CSF secretion with vector copy number in GM-CSF transduced human renal cell tumor cultures*

Five primary renal cell tumor cultures were plated at 3.5×10^6 cells in 225-cm^2 tissue culture flasks and transduced 24 h later with 25 ml of retrovirus containing supernatant in the presence of 10 μg/ml DEAE-dextran for 24 h. GM-CSF secretion was determined by enzyme-linked immunosorbent assay 48 h later and all values were normalized to ng/10^6 cells/24 h. The concentration of GM-CSF secretion was also determined for untransduced human renal carcinoma cultures at the same *in vitro* passage number. Vector copy number was determined by Southern blot analysis.

GM-CSF secretion pretransduction	GM-CSF secretion posttransduction	Vector copy no.	Histological subytpe
6.4	35	1.6	Grade II, clear cell
1.2	26	0.5	Grade II and III, clear cell and granular
2.5	48	2.0	Grade II, clear cell
3.4	74	0.9	Grade I with oncocytic features
4.7	63	0.7	Grade II with oncocytic features

A proliferating population of primary human tumor cells is critical for retroviral mediated transduction since proliferation of the majority of tumor cells within the culture is necessary to facilitate integration. For this reason, growth conditions necessary for *in vitro* expansion of several histologically different fresh human tumor explants were optimized. It is also worth noting that even the short-term culture of the primary tumor cells yielded a significant increase (greater than 10-fold) in the total number of tumor cells. In fact, of 26 fresh human renal cell explants received after nephrectomy, 21 of the 24 specimens (88%) with malignant histological subtypes were propagated in culture long enough to successfully undergo gene transfer.

Murine tumor vaccine studies have revealed that for GM-CSF a full antitumor immunization potential is obtained over a greater than 10-fold range of cytokine concentrations (7). However, immunization potential was extremely dependent on vaccinating cell dose, with increasing doses providing increased systemic protection against tumor challenge. It is therefore likely that at least 1×10^8 cytokine-secreting tumor cells will be needed to generate an optimal antitumor immune response in patients. Thus, to produce tumor vaccines from the majority of patient specimens, either 10 g of viable tumor must be available or the primary culture must be expanded at least 10-fold. Since the average weight of excised tumor specimens received by our laboratory is 2–3 g, in most cases vaccine development will depend on the success of *in vitro* expansion. Given that the majority of tumor cells proliferate for 2–3 passages under the growth conditions we have developed, it is unlikely that major populations of antigen-bearing cells will be selected out during the short-term culture period.

Our system has two further advantages over past approaches. First, we have found that it is possible to freeze and thaw previously transduced tumor cells without loss of cell viability and gene expression. This should allow for flexibility in therapy administration. Second, these cells can be irradiated following transduction, resulting in the inhibition of cell proliferation without loss of *in vitro* GM-CSF production. Our preclinical animal studies confirm the *in vivo* efficacy of these irradiated tumor vaccines (7). Thus, this vaccine should be as safe as it is effective.

The transduction efficiency ranged from 39 to 100%. This was particularly true when the vector was used to transfer the *LacZ* marker

gene. There are at least three possible explanations for variability. First, successful gene transfer may be dependent on the histological tumor type or the degree of cellular differentiation of the tumor cells that are being tranduced. Our data do not demonstrate a correlation of transduction efficiency with the histological cell type or degree of differentiation of the renal cell, pancreatic, and colon carcinomas that have been evaluated so far. In contrast, all of the ovarian carcinomas were from ascites, which may explain the less variable range of transduction efficiency among these more advanced populations of tumor cells. A comparison could not be made for the breast, lung, and squamous cell carcinomas because too few tumors of these histological types were evaluated. Second, integration of the transferred gene is dependent on proliferation of the tumor cell population. Therefore, efficient gene transfer requires proliferation of the majority of tumor cells within the explanted population. It may be that suboptimal tumor cell proliferation of some primary tumor cultures explains the wide range of transduction efficiencies among the initial gene transfer experiments performed using the *LacZ* marker gene. However, it does not account for the entire problem since we see less variation in transduction efficiency with improvement of our technique of retroviral supernatant collection. Third, transduction efficiency is dependent on the virus titer of retroviral supernatant, which will vary with different titer collections. This should no longer represent a significant practical problem as recent advances in long-term freezing of amphotropic retroviral supernatants will allow lots to be tested for titer prior to their use in vaccine preparation.[5]

In addition, when GM-CSF secretion was compared with the vector copy number for 5 genetically altered renal cell cultures, the number of integrated copies/cell did not perfectly correlate with the concentration of GM-CSF produced. A possible explanation for this is that expression of retrovirally transferred genes may be dependent on host cell-derived transcription factors that vary among different histologically similar tumor cultures. We also noted that with increasing passage of the human renal tumors cells, endogenous GM-CSF production was induced. However, levels of endogenous GM-CSF (which ranged from 0 to 20 ng/10^6 cells/24 h, with an average of 4.1 ng/10^6 cells/24 h) were far below the threshold for maximal vaccine potency (36 ng/10^6 cells/24 h) as determined in our animal studies.

In conclusion, we have shown that it is possible to establish and efficiently transduce short-term, primary human tumor cultures. The MFG retroviral vector system has made it technically feasible to provide safe, efficient gene therapy to patients with cancer. In addition, this vector appears flexible enough to transduce a wide variety of histological tumor types. We are planning to use this vector system in a Phase I study to evaluate the antitumor immune response generated by autologous GM-CSF-secreting renal tumor cells in patients in the near future.

References

1. Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., Karasuyama, H., Vogelstein, B., and Frost, P. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell, *60:* 397–403, 1990.
2. Golumbek, P. T., Lazenby, A. J., Levitsky, H. I., Jaffee, E. M., Karasuyama, H., Baker, M., and Pardoll, D. M. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. Science (Washington DC), *254:* 713–716, 1991.
3. Gansbacher, B., Zier, K., Daniels,,B., Cronin, K., Bannerji, R., and Gilboa, E. Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. J. Exp. Med., *172:* 1217–1224, 1990.
4. Tepper, R. I., Pattengale, P. K., and Leder, P. Murine interleukin-4 displays potent anti-tumor activity *in vivo*. Cell, *57:* 503–512, 1989.
5. Hock, H., Dorsch, M., Diamantstein, T., and Blankenstein, T. Interleukin 7 induces CD4+ T cell-dependent tumor rejection. J. Exp. Med., *174:* 1291–1298, 1991.
6. Asher, A. L., Mule, J. J., Kasid, A., Restifo, N. P., Salo, J. C., Reichert, C. M., Jaffe, G., Fendly, B., Kriegler, M., and Rosenberg, S. A. Murine tumor cells transduced with the gene for tumor necrosis factor-α. J. Immunol., *146:* 3227–3234, 1991.
7. Dranoff, G., Jaffee, E. M., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D. M., and Mulligan, R. C. Vaccination with irradiated tumor cells engineered to secrete murine GM-CSF stimulates potent, specific and long lasting anti-tumor immunity. Proc. Natl. Acad. Sci. USA, in press, 1993.
8. Porgador, A., Tzehoval, E., Katz, A., Vadai, E., Revel, M., Feldman, M., and Eisenback, L. Interleukin-6 gene transfection into Lewis lung carcinoma tumor cells suppresses the malignant phenotype and confers immunotherapeutic competence against parental metastatic cells. Cancer Res., *52:* 3679–3686, 1992.
9. Colombo, M. P., Ferrari, G., Stoppacciaro, A., Parenza, M., Rodolfo, M., Mavilio, F., and Parmiani, G. Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma *in vivo*. J. Exp. Med., *173:* 889–897, 1991.
10. Gastl, G., Finstad, C. L., Guarini, A., Bosl, G., Gilboa, E., Bander, N. H., and Gansbacher, B. Retroviral vector-mediated lymphokine gene transfer into human renal cancer cells. Cancer Res., *52:* 6229–6236, 1992.
11. Rill, D. R., Buschle, M., Foreman, N. K., Bartholomew, C., Moen, R. C., Santana, V. M., Ihle, J. N., and Brenner, M. K. Retrovirus-mediated gene transfer as an approach to analyze neuroblastoma relapse after autologous bone marrow transplantation. Hum. Gene Ther., *3:* 129–136, 1992.
12. Degiovanni, G., Lahaye, T., Herin, M., Hainaut, P., and Boon, T. Antigenic heterogeneity of a human melanoma tumor detected by autologous CTL clones. Eur J. Immunol., *18:* 671–676, 1988.
13. Knuth, A., Wolfel, T., Klegmann, E., Boon, T., and Meyer zum Buschenfelde, K-H. Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. Proc. Natl. Acad. Sci. USA, *86:* 2804–2808, 1989.
14. Band, V., and Sager, R. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. Proc. Natl. Acad. Sci. USA, *86:* 1249–1253, 1989.
15. Williams, R. D., Elliott, A. Y., Stein, N., and Fraley, E. E. *In vitro* cultivation of human renal cell cancer. I. Establishment of cells in culture. In Vitro (Rockville), *12:* 623–627, 1976.
16. Armentano, D., Sheau-Fung, Y., Kantoff, P., von Ruden, T., Anderson, W. F., and Gilboa, E. Effect of internal viral sequences on the utility of retroviral vectors. J. Virol., *61:* 1647–1650, 1987.
17. Danos, O., and Mulligan, R. C. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA, *85:* 6460–6464, 1988.
18. Lojda, Z. Indigogenic methods for glycosidases. II. An improved method for β-D-galactosidase and its application to localization studies of the enzymes in the intestine and in other tissues. Histochemie, *23:* 266–288, 1970.
19. Kitamura T., Tojo A., Kuwaki T., Chiba S., Miyazono K., Urabe A., and Takaku F. Identification and analysis of human erythropoietin receptors on a factor-dependent cell line, TF-1. Blood, *73:* 375–380, 1989.
20. Wilson, J. M., Jefferson, D. M., Chowdhury, J. R., Novikoff, P. M., Johnston, D. E., and Mulligan, R. C. Retrovirus-mediated transduction of adult hepatocytes. Proc. Natl. Acad. Sci. USA, *85:* 3014–3018, 1988.